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Enzymatic Removal and Disinfection of Bacterial Biofilms

CHARLOTTE JOHANSEN,[1]* PER FALHOLT,[1] AND LONE GRAM[2]

Enzyme Development and Applications, Novo Nordisk A/S, Novo Alle, DK-2880 Bagsværd,[1] and Danish Institute for Fisheries Research, Department of Seafood Research, Technical University of Denmark, DK-2800 Lyngby,[2] Denmark

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Model biofilms of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas fluorescens*, and *Pseudomonas aeruginosa* were made on steel and polypropylene substrata. Plaque-resembling biofilms of *Streptococcus mutans*, *Actinomyces viscosus*, and *Fusobacterium nucleatum* were made on saliva-coated hydroxyapatite. The activity of enzymes against bacterial cells in biofilm was measured by fluorescence microscopy and an indirect conductance test in which evolution of carbon dioxide was measured. Glucose oxidase combined with lactoperoxidase was bactericidal against biofilm bacteria but did not remove the biofilm from the substrata. A complex mixture of polysaccharide-hydrolyzing enzymes was able to remove bacterial biofilm from steel and polypropylene substrata but did not have a significant bactericidal activity. Combining oxidoreductases with polysaccharide-hydrolyzing enzymes resulted in bactericidal activity as well as removal of the biofilm.

In nutrient-limited ecosystems, such as the aquatic environment, bacteria have a marked tendency to attach to surfaces and initiate the formation of a biofilm (8, 12), causing problems such as increased frictional resistance to fluids in water conduits and on ship hulls (fouling) (21), decreased heat transfer from heat exchangers (7), corrosion of metallic substrata (20), and contamination in the food and biotechnology industries (7). Biofilms are also a severe problem in medical science and industry, causing dental plaque (13), contaminated endoscopes and contact lenses (28), prosthetic device colonization, and biofilm formation on medical implants (24).

The biofilm matrix is a collection of microcolonies with water channels in between and an assortment of cells and extracellular polymers (polysaccharides, glycoproteins, and proteins) (5, 6, 8, 11). Bacterial extracellular polysaccharides are composed of homo- and heteropolysaccharides of, in particular, glucose, fucose, mannose, galactose, fructose, pyruvate, and mannuronic acid- or glucuronic acid-based complexes (3). The different types of bonds between the saccharides give rise to a multitude of different polysaccharides, including levans, polymannans, dextrans, cellulose, amylopectin, glycogen, and alginate.

Enzymes can be used for degradation of biofilm (1, 3, 29, 31), but due to the heterogeneity of the extracellular polysaccharides in the biofilm, a mixture of enzyme activities may be necessary for a sufficient degradation of bacterial biofilm.

The purpose of this study was to assess the applicability of commercially available enzymes for removal of bacterial biofilm as well as for their bactericidal activity against cells in biofilm.

Formation and enzyme treatments of biofilms. Stainless steel type AISI 304 with a no. 4 finish (polish grain 180) was cut into 12- by 20-mm discs. The discs were cleaned in water, followed by cleaning with chloroform, methanol, and finally acetone (5 min each) before sterilization by autoclaving at 121°C for 20 min prior to use. Polypropylene discs (12 by 20 mm; Ral. 7032, Dukadan A/S) were cleaned by scrubbing in a neutral detergent (Triton) and then rinsed in water before autoclaving.

Sterile steel or polypropylene discs were clamped vertically in a sterile steel rack in a beaker. The rack holds up to 20 discs in an arrangement which, when immersed in culture medium, allows the free circulation of liquid. *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 10148, and *Pseudomonas fluorescens* AH2 (14) were precultured in tryptone soy broth (TSB) (Oxoid CM129) for 24 h at 26°C. *Staphylococcus epidermidis* DSM 20042 was precultured in TSB for 24 h at 30°C. *Staphylococcus* spp. were inoculated in TSB and *Pseudomonas* spp. were inoculated (approximately 10^3 CFU/ml) in TSB diluted 1:5 with sterile water. The inoculated medium was poured into the beaker, covering the discs, and a biofilm was allowed to develop on both sides of the discs at 26°C (*S. epidermidis* at 30°C) over 4 days with slow stirring (200 rpm).

All discs were aseptically rinsed for 1 min in sterile phosphate buffer (0.067 M; pH 7) to remove poorly attached cells before incubation with enzymes (Table 1) in phosphate buffer at 20°C for 15 min without agitation. Sterile buffer with no enzymes added was used as a control.

Glucose oxidase was used with 3 g of D-(+)-glucose (Sigma G-7528) per liter as the electron donor and with oxygen as the electron acceptor being reduced to hydrogen peroxide. Lactoperoxidase was used with hydrogen peroxide as the electron acceptor and 2 mM thiocyanate (Merck) as the electron donor (10, 27). Pectinex Ultra SP is a multicomponent enzyme preparation containing protease activity and a wide range of carbohydrases, including pectinase, arabanase, cellulase, hemicellulase, β-glucanase, and xylanase activities.

The bactericidal activities of glucose oxidase and lactoperoxidase on planktonic cells of *S. aureus*, *S. epidermidis*, *P. aeruginosa*, and *P. fluorescens* were also determined. Planktonic cells from the biofilm development were diluted 1:9 in 0.067 M phosphate buffer (pH 7.0) and mixed with glucose oxidase (0, 5, or 10 glucose oxidase units [GODU]/ml) and lactoperoxidase (0, 1, or 5 U/ml) at 20°C for 15 min. Bacterial counts were estimated by inoculation of Malthus tubes containing growth medium (see below) with 0.1 ml of cell suspension.

Bacteriological problems with contact lenses are primarily caused by the attachment of a thin layer of bacteria to the lens surface. *P. aeruginosa* was grown in TSB (18 h; 26°C), harvested by centrifugation at 2,000 × *g* for 10 min, and suspended in 0.067 M phosphate buffer (pH 7) to approximately 10^4 CFU/ml. Bacteria were allowed to attach by immersing the contact lenses (73% water; Sterile Rythmic, Essilor, France) in the bacterial suspension for 5 h at 20°C, after which the contact lenses were incubated with enzymes (Table 1) at 20°C for 5 min without agitation.

Oral plaque-resembling biofilm was made with *Streptococcus*

* Corresponding author. Mailing address: Novo Nordisk A/S, Novo Alle 1A.1.34, DK-2880 Bagsværd, Denmark. Phone: 45 44 42 14 31. Fax: 45 44 44 42 33. E-mail: cjoh@novo.dk.

TABLE 1. Enzymes tested for activity against bacterial cells in biofilm

Substratum and species	Enzyme	Activity
Stainless steel and polypropylene	Glucose oxidase (Novo Nordisk A/S; SP541)	2,000 GODU/g
Staphylococcus aureus	Lactoperoxidase (Sigma L-2005)	98,000 U/g
Staphylococcus epidermidis	Pectinex Ultra SP (Novo Nordisk A/S)	8,800 PSU/ml
Pseudomonas aeruginosa		
Pseudomonas fluorescens		
Hydroxyapatite	Mutanase (Novo Nordisk A/S; SP538)	30 MU/ml
Streptococcus mutans	Dextranase (Novo Nordisk A/S; SP821)	37 kDU/ml
Actinomyces viscosus		
Fusobacterium nucleatum		
Soft contact lenses	Glucose oxidase (Novo Nordisk A/S; SP541)	2,000 GODU/g
Pseudomonas aeruginosa	Subtilisin A (Novo Nordisk A/S; SP544)	29.3 AU/g[a]

[a] AU, subtilisin A units.

mutans SFAG, CBS 350.71, *Actinomyces viscosus* DSM 43329, and *Fusobacterium nucleatum* subsp. *polymorphum* DSM 20482. Precultures were grown anaerobically at 37°C in brain heart infusion broth (BHI) (Oxoid CM225). Anaerobic conditions were obtained by flushing the substrate with nitrogen immediately before autoclaving. The anaerobic substrate was inoculated with a syringe through a rubber stopper. *F. nucleatum* subsp. *polymorphum* DSM 20482 was grown anaerobically at 37°C in BHI containing sterile Na_2S (pH 7.0), which was added immediately before inoculation at a final concentration of 5 g/liter. Hydroxyapatite discs (diameter, 12 mm) were sterilized by autoclaving and coated for 18 h at 37°C with filter-sterilized saliva collected from one person.

Saliva-coated hydroxyapatite discs were placed in a sterile rack in a beaker. BHI broth containing 0.2% sucrose was poured into the beaker, covering the discs. Sterile Na_2S (pH 7.0) was added at a final concentration of 5 g/liter immediately before inoculation. A 1:1:1 mixture of *Streptococcus mutans*, *A. viscosus*, and *F. nucleatum* grown anaerobically (BHI; 37°C; 24 h) was used as the inoculum at a concentration of approximately 10^6 CFU/ml. The discs were incubated at 37°C for 4 days with slight stirring (100 rpm). Anaerobic conditions were obtained with an anaerobic culture jar with a gas generating kit (Oxoid anaerobic system BR38). Mutanase with α-1,3-glucanase activity and dextranase-hydrolyzing α-1,6-D-glucosidic linkages in dextran were used for plaque removal (Table 1). Hydroxyapatite discs were incubated with enzymes in acetate buffer (0.02 M; pH 5.5) (22) at 20°C for 2 min with agitation.

After enzyme treatment, the different substrata were gently rinsed once in sterile buffer prior to enumeration of bacteria by microscopy or by conductance measurements (see below).

Methods used for the evaluation of biofilms. Fluorescence microscopy was used to evaluate the number of total and respiring bacterial cells in biofilms. The tetrazolium salt 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Polysciences, Inc., Warrington, Pa.) was used as an indicator of cellular viability (26). The DNA-binding fluorochrome DAPI (4′,6-diamidino-2-phenylindole; Sigma D-9542) was used as an indicator for the total cell number, and the biofilm cells were stained with DAPI, after CTC staining, to allow enumeration of total and respiring cells within the same preparation (26). The stained cells were examined with the ×100 oil immersion fluorescence objective lens on an Olympus model BX50 microscope equipped with a 200 W mercury lamp. The filter combination used for viewing CTC-stained cells was a 480- to 550-nm excitation filter and a 590-nm barrier filter (Olympus cube model U-MSWG). DAPI-stained cells were viewed with a 330- to 385-nm excitation filter and a 420-nm barrier filter (Olympus cube model U-MWU).

The effect of enzymes on thick biofilms could easily be evaluated by microscopy; however, evaluations of the effect of enzymes on thin films was difficult. Therefore, the estimation of exact cell number in all experiments was determined by an indirect conductance measurement (2, 9, 17, 23) with the Malthus Flexi 2000 instrument (Malthus Instrument Limited, West Sussex, United Kingdom). The substratum with biofilm cells was, after enzyme treatment, transferred to a Malthus glass tube containing growth medium. Growth of biofilm cells results in the evolution of carbon dioxide, which will diffuse into an inner tube containing potassium hydroxide, placed inside the Malthus glass tube. The neutralization of potassium hydroxide will result in a change in conductance, which is measured by electrodes immersed in the alkaline solution.

TSB was used as the growth medium for detection of *P. aeruginosa* and *P. fluorescens*, whereas BHI was used for detection of *S. aureus* and *Streptococcus mutans.*

Changes in conductance, caused by carbon dioxide metabolism, were plotted against time, and the detection time was determined as the time taken from the start of the measurement until a rapid change in conductance was detectable by the Malthus instrument. The detection time can be related to the number of cells present at the start of the test by use of a calibration curve, which was constructed for each organism by inoculating Malthus tubes with a 10-fold-dilution series of the planktonic cells from the biofilm culture (16, 17). As control experiments, discs with biofilm that were not treated with enzymes were incubated in the Malthus tubes together with the different enzyme solutions (Table 1) to exclude interference of the enzymes with the carbon dioxide metabolism of the biofilm cells.

For a quantitative determination of viable counts in plaque, a 10-fold-dilution series was prepared from the mixed culture and a calibration curve was made under aerobic conditions whereby only growth of *Streptococcus mutans* was measured. After incubation with enzymes, the plaque discs were rinsed in the same buffer as the one used for the enzyme treatment, transferred into the Malthus tubes, and incubated aerobically at 37°C. Thus, enzymatic plaque removal was quantitatively determined with respect to the removal of *Streptococcus mutans.*

By the Malthus method it is not possible to distinguish between a bactericidal activity of the enzymes and an enzymatic removal of biofilm. Also, sublethally injured cells may prolong the detection time and result in underestimation of the number of living biofilm cells. Therefore, a decrease in living bacteria

TABLE 2. Cell number present on stainless steel after treatment with Pectinex Ultra SP[a]

Pectinex Ultra (PSU/ml)	Cell number ($\log_{10}$ CFU/disc)			
	S. aureus	*S. epidermidis*	*P. aeruginosa*	*P. fluorescens*
0	7.4×10^6	4.5×10^7	1.8×10^8	2.4×10^8
0.18	1.3×10^6	2.7×10^7	1.0×10^8	2.9×10^8
1.8	1.5×10^5	2.5×10^7	1.4×10^7	8.9×10^7
18	1.4×10^5	3.2×10^6	9.0×10^6	7.0×10^7
180	6.7×10^4	2.3×10^6	9.0×10^6	2.5×10^7
1,800	2.2×10^4	1.4×10^6	8.9×10^6	2.1×10^7

[a] Cells were treated with Pectinex Ultra SP for 15 min at 20°C (pH 7). Results shown are averages of the results of two independent experiments ($n = 4$).

on the substrata has to be compared with the simultaneous removal of biofilm from the substrata, which was estimated by DAPI and CTC staining.

The influence of Pectinex Ultra was analyzed by Duncan's multiple-range test. The influence and interactions of the other enzymes were analyzed by multiple regression analyses with a factorial design (Statgraphics version 7 plus; Statistical Graphic Corporation, Inc., Rockville, Md.).

Effects of enzymes on biofilms. In general, the biofilms of *S. aureus* and *S. epidermidis* were thin and by microscopy appeared as microcolonies on the surfaces, whereas the biofilms of *Pseudomonas* spp. appeared as thick films with large colonies and water channels. Pectinex Ultra reduced the number of bacterial cells in biofilms on stainless steel (Table 2). The activity of Pectinex Ultra was manifested as a removal of biofilm without any significant bactericidal activity against either of the four strains, as determined by combined DAPI and CTC staining. DAPI-stained untreated *P. aeruginosa* biofilm on steel (Fig. 1A) showed the total cell number, and cells stainable by DAPI were also stained with CTC, indicating that all visible cells were respiring. After treatment with Pectinex Ultra, DAPI staining clearly showed a removal of *P. aeruginosa* biofilm from the surface (Fig. 1B) and CTC staining showed the remaining cells to be respiring, which indicate that Pectinex Ultra is not bactericidal. Thus, the activity of Pectinex Ultra is mainly a degradation of extracellular polysaccharides. In general, *S. aureus* and *S. epidermidis* biofilms were more sensitive to enzymatic removal by Pectinex Ultra than *P. aeruginosa* and *P. fluorescens* biofilms (Table 2). *S. aureus* biofilm was most sensitive to Pectinex Ultra, as 1.8 Pectinex Ultra SP units (PSU) of Pectinex Ultra per ml decreased the cell number on

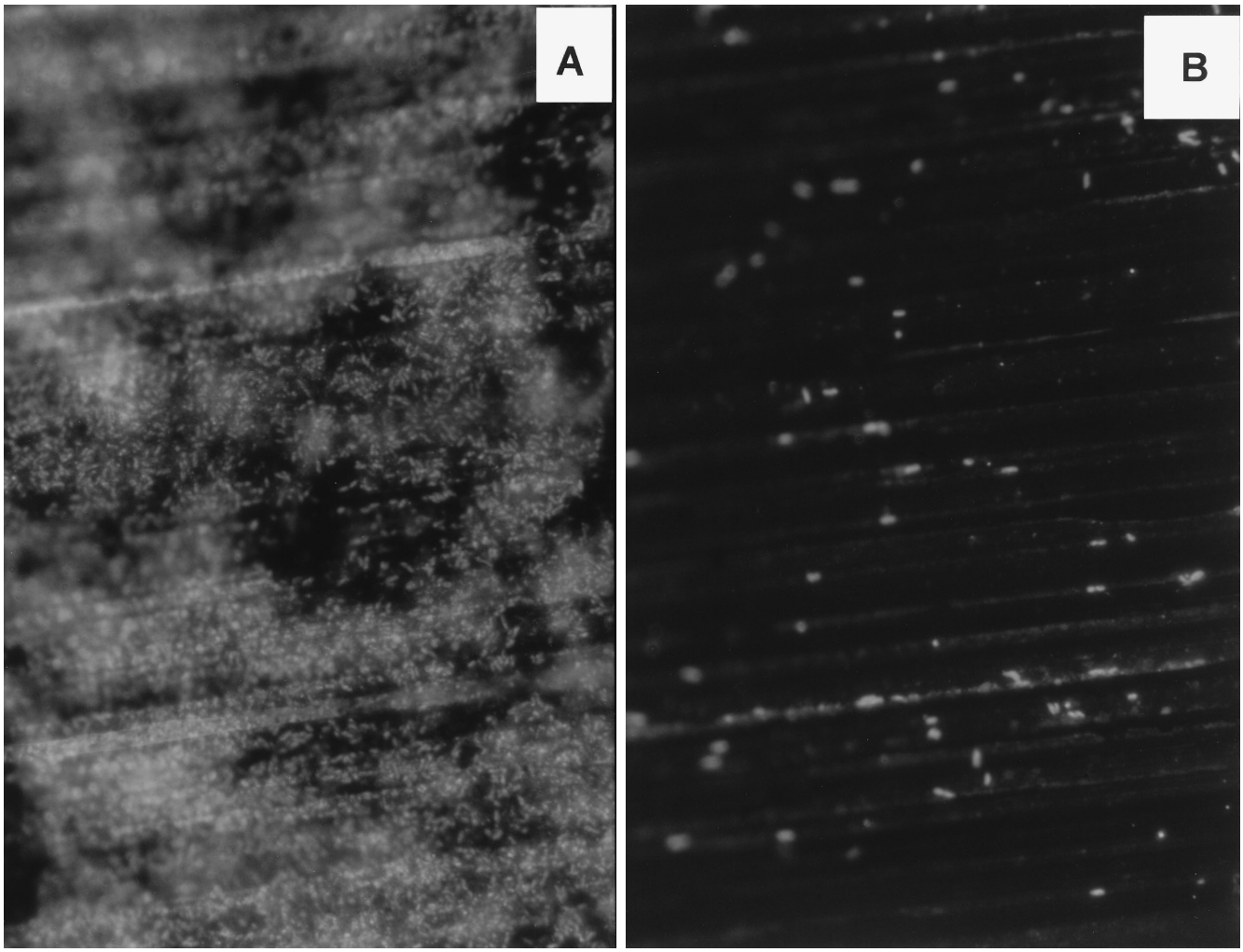

FIG. 1. Fluorescence microscopy of a 4-day biofilm of *P. aeruginosa* on stainless steel. (A) Total biofilm cells present in control without enzymatic treatment; (B) staining after Pectinex Ultra treatment (1.8 PSU/ml) for 15 min.

TABLE 3. Bactericidal activity of glucose oxidase and lactoperoxidase against *P. aeruginosa* and *P. fluorescens*[a]

Glucose oxidase (GODU/ml)	Lactoperoxidase (U/ml)	Bactericidal activity (log_{10} reduction)			
		P. aeruginosa		*P. fluorescens*	
		Biofilm cells at 1.7×10^8 CFU/disc	Planktonic cells at 2.3×10^8 CFU/ml	Biofilm cells at 1.9×10^8 CFU/disc	Planktonic cells at 8.0×10^8 CFU/ml
0	0				
0	1	0	0.8	0.1	0.9
0	5	0	1.1	0.9	1.1
5	0	0	0	0.8	0.1
5	1	1.5	2.5	2.2	2.7
5	5	1.7	3.4	2.5	4
10	0	0.3	0	2.4	0.2
10	1	3	2.7	3	3
10	5	3	3.5	3	4.5

[a] Biofilm cells on stainless steel and planktonic cells were treated for 15 min at 20°C. The cell concentration before enzyme treatment is given, and bactericidal activity is shown relative to the cell numbers for untreated samples ($n = 3$).

the substrata more than 1 log unit. *P. fluorescens* was the most resistant biofilm, as 1,800 PSU of Pectinex Ultra per ml was needed to decrease the number of biofilm cells by 1 log unit. This difference may be due to the thinner biofilm obtained with *Staphylococcus* spp. or variations in the composition of the extracellular polymers in the biofilm. The sensitivity of biofilm cells on and the removal of biofilm from polypropylene by Pectinex Ultra were similar to the sensitivity of biofilm cells on and the removal of biofilm from stainless steel (data not shown).

The combination of glucose oxidase and lactoperoxidase significantly lowered the counts of actively respiring cells in the four tested biofilms (Table 3 and 4). The *Staphylococcus* biofilm was reduced 1 to 2 log units when exposed to glucose oxidase (10 GODU/ml) and lactoperoxidase (5 U/ml), whereas the *Pseudomonas* biofilm was reduced more than 3 log units. The extent of killing was, however, lower than that obtained when planktonic suspensions of cells had been exposed, as

TABLE 4. Bactericidal activity of glucose oxidase and lactoperoxidase against *S. aureus* and *S. epidermis*[a]

Glucose oxidase (GODU/ml)	Lactoperoxidase (U/ml)	Bactericidal activity (log_{10} reduction)			
		S. aureus		*S. epidermidis*	
		Biofilm cells at 3.1×10^7 CFU/disc	Planktonic cells at 7.0 $\times 10^7$ CFU/ml	Biofilm cells at 3.6×10^7 CFU/disc	Planktonic cells at 2.2 $\times 10^6$ CFU/ml
0	0				
0	1	0	0	0.2	0
0	5	0	0	0.4	0.3
5	0	0	0	0.3	0.6
5	1	0.5	0.4	0.9	0.8
5	5	0.7	1	1.2	2.3
10	0	0	0.1	0.2	1.2
10	1	2	2.3	1.2	4
10	5	2	2.7	1.4	5

[a] Biofilm cells on stainless steel and planktonic cells were treated for 15 min at 20°C. The cell concentration before enzyme treatment is given, and bactericidal activity is shown relative to the cell numbers for untreated samples ($n = 3$).

planktonic cells of *Pseudomonas* spp. were reduced approximately 5 log units when exposed to glucose oxidase (10 GODU/ml) and lactoperoxidase (5 U/ml) (Table 3). Planktonic cells of *S. aureus* were comparable to biofilm cells in sensitivity to oxidoreductases; thus, *S. aureus* was reduced approximately 2 to 3 log units when exposed to glucose oxidase (10 GODU/ml) and lactoperoxidase (5 U/ml) (Table 4). Planktonic cells of *S. epidermidis* were significantly more sensitive to oxidoreductases than the biofilm cells, as the number of viable planktonic cells decreased approximately 5 log units compared to a reduction of 1 log unit in the number of biofilm cells.

There was no significant difference in the bactericidal activity of the oxidoreductase system towards biofilm on stainless steel compared to biofilm on polypropylene, except for *P. aeruginosa* biofilm on polypropylene, where glucose oxidase (5 GODU/ml) combined with lactoperoxidase (5 U/ml) killed 99.99% of the biofilm cells compared to 98% of the *P. aeruginosa* biofilm cells on stainless steel (data not shown).

Our data thus confirm that biofilm cells are more resistant than planktonic cells (4, 18), and this is believed to be caused by physical protection by the biofilm matrix or by an altered physiology of bacterial cells in the biofilm mode of growth. In some experiments, bactericidal activity by either glucose oxidase or lactoperoxidase alone was observed; however, the susceptibility of the biofilm cells varied from day to day, which may be explained by differences in, e.g., catalase activity and oxygen concentration in the biofilm. Thus, biofilm cells in anaerobic environments may escape inhibition by oxidoreductases, even though the cells have limited resistance to oxidoreductases under aerobic conditions (30). Also, the diffusion of thiocyanate and hydrogen peroxide into the biofilm will decrease the susceptibility of biofilm cells compared to planktonic cells, suggesting that the underlying cells in the biofilm will escape the bactericidal activity of the oxidoreductases unless the biofilm cells are released from the surface. This may explain the small difference in susceptibility between *S. aureus* biofilm and planktonic cells, as the thin *Staphylococcus* biofilm will provide limited protection of the biofilm cells compared to the thick biofilms of *Pseudomonas* spp.

Glucose oxidase (5 GODU/ml) decreased the number of respiring attached *P. aeruginosa* cells on contact lenses from 3.0×10^5 CFU/ml to 1.1×10^4 CFU/ml, and the bactericidal activity of glucose oxidase may be further increased by decreasing the pH to 5 to 6 or by combining the glucose oxidase with lactoperoxidase (data not shown). Subtilisin A, which was used for removal of protein, had no significant bactericidal activity on the attached *P. aeruginosa* cells. In addition, subtilisin A completely inhibited the bactericidal activity of glucose oxidase.

One of the primary routes of ocular infections is transfer of *P. aeruginosa* from a contaminated lens case via the lens or fingers (19), and a disinfectant, therefore, has to be effective against biofilm in the lens case as well as cells attached to the lenses. We have found that the glucose oxidase and lactoperoxidase system causes a 4- to 5-log reduction in *P. aeruginosa* biofilm on polypropylene. However, this reduction in respiring biofilm cells may not be sufficient in case of a heavily biofilm-contaminated lens case.

The plaque biofilm appeared as a thick film with a high cell density compared to the other biofilms. After enzyme treatment, the number of bacteria on the saliva-treated hydroxyapatite surface was significantly reduced. Dextranase (1 kilodextranase unit [kDU]/ml) decreased the cell number on the hydroxyapatite discs by approximately 1.5 log units, whereas mutanase (1 mutanase unit [MU]/ml) reduced the cell number by approximately 0.5 log units (Fig. 2). The combination of mutanase

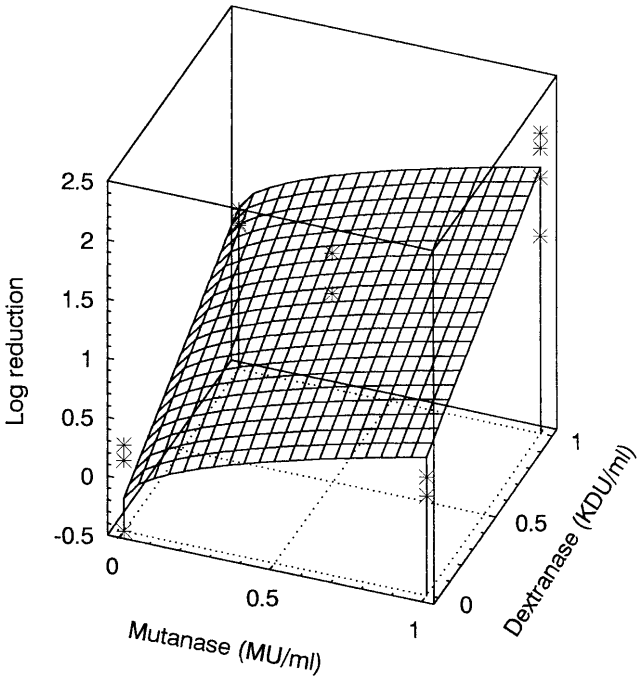

FIG. 2. Reduction in biofilm on saliva-coated hydroxyapatite discs caused by dextranase and mutanase. The response regression ($r = 0.85$), response surface plot was calculated as follows: log reduction = $0.119 + 0.639 \times$ (kDU of dextranase/ml) $+ 1.03 \times [$(MU of mutanase/ml) $\times$ (kDU of dextranase/ml)$]^{0.5}$.

and dextranase resulted in an additive effect (Fig. 2), as the total cell number on the hydroxyapatite discs after treatment with mutanase (1 MU/ml) and dextranase (1 kDU/ml) decreased from approximately 10^8 CFU/disc to approximately 10^6 CFU/disc.

Mutanase and dextranase were shown to remove oral plaque from hydroxyapatite but were not bactericidal. However, in the case of enzymatic plaque removal, a complete elimination of the biofilm will be undesirable, as the plaque organisms are part of the natural oral microbial population.

Conclusions. The application of enzymes for control of protein biofilm on surfaces and in closed pipelines is well known (1, 3, 31). In particular, proteases are used in pipelines and for removal of protein from contact lenses (25). The use of enzymes for removal of bacterial biofilm is still limited, partly due to the very low prices of the chemicals in use. Also, the lack of techniques for quantitative evaluation of the effect of enzymes, as well as the commercial accessibility of different enzyme activities, limits their usage. It is known that monocomponent enzymes can be used for biofilm removal (1, 15, 31). However, the heterogenicity of the biofilm matrix limits the potential of monocompound enzymes, and one advantage of Pectinex Ultra is its wide range of enzyme activities, which makes it useful for removal of complex biofilms. Oxidoreductases were bactericidal against biofilm cells but did not cause removal of the biofilm. Therefore, the combination of polysaccharide-hydrolyzing enzymes and oxidoreductases caused both removal and inactivation of the bacterial biofilms.

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